

Remarks

Claims 46-66 are pending in the subject application. Applicants acknowledge that claims 47-64 and 66 have been withdrawn from further consideration as being drawn to a non-elected invention. By this Amendment, Applicants have canceled claims 46-66 and added new claims 67-71. Support for the new claims can be found throughout the subject specification and in the claims as originally filed. Entry and consideration of the amendments presented herein is respectfully requested. Accordingly, claims 67-71 are currently before the Examiner. Favorable consideration of the pending claims is respectfully requested.

Claim 46 is objected to for reciting non-elected subject matter. By this Amendment, the claim has been canceled thereby rendering this objection moot. Accordingly, reconsideration and withdrawal of the objection is respectfully requested.

Claims 46 and 65 are rejected under 35 U.S.C. §101 and under 35 U.S.C. §112, first paragraph, on the grounds the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility. The Office Action argues that the claimed invention lacks patentable utility and is not enabled on the basis that the application fails to provide any specific disclosure that the claimed polypeptide modulates the activity of any protein. The Office Action further argues that the asserted utility of the claimed polypeptide (as an antagonist of midkine) is hypothetical and based upon a bioinformatics approach. The Office Action also argues that the art generally acknowledges that the asserted antagonistic function of the claimed protein (disclosed at page 6, lines 14-19) cannot be predicted on structural similarity to a protein in the sequence databases (citing to Skolnick *et al.*) and/or that the disclosure's teaching that the deletion of the C-terminus of pleiotrophin is known in the art to interfere with receptor binding. Applicants respectfully assert that the claimed invention has substantial utility and, therefore, is enabled and traverse the rejection. Reconsideration and withdrawal of the rejections is respectfully requested.

Turning, first, to the Patent Office's argument pertaining to the "speculated" role of the claimed polypeptide as an antagonist of midkine, Applicants respectfully submit that statements made by the applicant in the specification cannot, standing alone, be the basis for a lack of utility rejection under 35 U.S.C. 101 or 35 U.S.C. 112. *Tol-O-Matic, Inc. v. Proma Produkt-Und Mktg. Gesellschaft m.b.h.*, 945 F.2d 1546, 1553, 20 USPQ2d 1332, 1338 (Fed. Cir. 1991). Further,

asserted utilities of an invention carry a presumption of enablement and/or utility and the Patent Office must make a *prima facie* showing that the claimed invention lacks utility and is not enabled in establishing a rejection under 35 U.S.C. §§101 and 112, first paragraph and the Patent Office must also articulate the factual assumptions and provide evidentiary support relied upon in establishing the *prima facie* showing. In this case, the Office Action has provided no basis as to why one skilled in the art would not have accepted the asserted utility of the claimed polypeptide as a midkine antagonist in view of the teachings provided in the as-filed specification; rather, the Office Action relies on an argument that the identification of the claimed polypeptide as a midkine antagonist on the basis of a bioinformatics approach is simply insufficient to support the asserted utility of the claimed polypeptide as an midkine antagonist or as interfering with midkine binding to protein tyrosine phosphatase ζ . Applicants respectfully submit that the articulated arguments are insufficient to support a rejection of the claimed invention and request reconsideration and withdrawal of the rejections.

Further, the asserted utility of the claimed polypeptide as a midkine antagonist is discussed in the as-filed specification. For example, specific annotation allowed the functional characterization of INSP106 and, as explained at page 6, lines, 14-19, the particular structure of INSP106 allowed Applicants to deduce the specific and credible function as a dominant negative antagonist to midkine. Midkine acts as a dimer in a head to head fashion involving both the N and C domains and binds to a protein tyrosine phosphatase ζ . The proline rich tail is suggested to interfere with receptor binding when INSP106 dimerizes with functional midkine proteins (as shown in Figure 3 of the application) thus leading to the dominant negative effect of INSP106 (*e.g.*, interference with binding to protein tyrosine phosphatase ζ). Such mechanisms of action (dominant negative effects) are art recognized as is evidenced by Bernard-Pierrot *et al.* (*J. Biol. Chem.*, 2002, 277(35):32071-32077—cited in the international search report) where deletion of the C-terminus of heparin affin regulatory peptide (HARP or pleiotrophin) results in interference with receptor binding in a manner similar to that disclosed herein (see Figure 3 of the as-filed application and page 32076, column 1, paragraph 3) and Zhang *et al.* (*PNAS*, 1999, 96:6734-6738; copy attached for the Examiner's convenience). Thus, it is respectfully submitted that one skilled in the art would have reasonably believed, more likely than not, that the claimed polypeptide was capable of antagonizing midkine function by interfering

with receptor binding as was asserted within the as-filed specification. Accordingly, reconsideration and withdrawal of the rejections is respectfully requested.

Claim 46 is rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Office Action argues that the as-filed specification fails to provide adequate written description for the claimed fragments, functional equivalents and antigenic determinants of SEQ ID NO:2 and that the claims do not require any conserved structure or other disclosed distinguishing feature. Applicants respectfully assert that there is adequate written description in the subject specification to convey to the ordinarily skilled artisan that they had possession of the claimed invention; however, in the interest of advancing prosecution in this matter, fragments, functional equivalents and antigenic determinants of SEQ ID NO: 2 are no longer claimed. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Claims 46 and 65 are rejected under 35 U.S.C. § 102(e) as anticipated by Tang *et al.* (WO 03/025142). The Office Action indicates that Tang *et al.* describe an isolated polypeptide specifically comprising an amino acid sequence that is 97.8% identical to SEQ ID NO: 2 and specifically excludes the amino acid sequence of SEQ ID NO: 10 of the subject claims. Applicants respectfully assert that the Tang *et al.* reference does not anticipate the claimed invention as it fails to teach the claimed polypeptide. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. § 102(e) is respectfully requested.

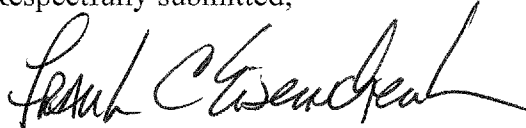
It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicants' agreement with or acquiescence in the Examiner's position. Applicants expressly reserve the right to pursue the invention(s) disclosed in the subject application, including any subject matter canceled or not pursued during prosecution of the subject application, in a related application.

In view of the foregoing remarks and amendments to the claims, Applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,

A handwritten signature in black ink, appearing to read 'Frank C. Eisenschenk', written in a cursive style.

Frank C. Eisenschenk, Ph.D.

Patent Attorney

Registration No. 45,332

Phone No.: 352-375-8100

Fax No.: 352-372-5800

Address: P.O. Box 142950
Gainesville, FL 32614-2950

FCE/sl

Attachment: Zhang *et al.*, 1999

A dominant-negative pleiotrophin mutant introduced by homologous recombination leads to germ-cell apoptosis in male mice

NAN ZHANG, HSIU-JENG YEH, RONG ZHONG, YUE-SHENG LI*, AND THOMAS F. DEUEL†

Division of Growth Regulation, Beth Israel Deaconess Medical Center at Harvard Medical School, 41 Avenue Louis Pasteur, Boston, MA 02215

Communicated by David M. Kipnis, Washington University School of medicine, St. Louis, MO, March 3, 1999 (received for review December 28, 1998)

ABSTRACT Pleiotrophin (PTN) is an 18-kDa heparin-binding secretory growth/differentiation factor for different cell types. Its gene is differentially expressed in both mesenchyme and central nervous system during development and highly expressed in a number of different human tumors. Recently, a PTN mutant was found to act as a dominant-negative effector of PTN signaling. We have now used homologous recombination to introduce the dominant-negative PTN mutant into embryonic stem cells to generate chimeric mice. All highly chimeric male mice with germinal epithelium exclusively derived from embryonic stem cells with the heterologous PTN mutation were sterile. Their testes were uniformly atrophic, and the spermatocytes were strikingly apoptotic at all stages of development. The results support a central role of PTN signaling in normal spermatogenesis and suggest that interruption of PTN signaling may lead to sterility in males.

Pleiotrophin (PTN) (1, 2), also known as HB-GAM (3), is a heparin-binding protein of 168 aa with a 32-aa signal sequence (2). It was purified from bovine uterus (1) as a mitogen for murine fibroblasts and from neonatal rat brain (1, 3) as a neurite outgrowth promoter. Transcripts of the *Ptn* gene are differentially expressed in both mesenchyme and cells of the central nervous system during mouse development (2, 4, 5), whereas stable but significantly lower levels of PTN gene expression limited to fewer cell types are seen in adults (6). An exception in adults is found in regenerating tissues in which PTN transcripts are sharply increased in specific cell types, for example, in endothelial cells, macrophages, and activated astrocytes after ischemic injury in brain and in cells stimulated by platelet-derived growth factor (7, 8).

PTN is also a mitogen for brain capillary endothelial cells (8–10) and SW-13 (adrenal carcinomas) cells (10) and is angiogenic in the rabbit corneal pocket assay (8, 9). PTN induces differentiation responses and neoantigen expression in oligodendrocyte progenitors (H.-J.Y. and T.F.D., unpublished data), is expressed in bone (11, 12), and stimulates proliferation and induction of differentiation markers in osteoblasts in culture (13).

The human, bovine, rat, and mouse *Ptn* cDNAs predict extraordinarily conserved mature proteins of 136 aa with clusters of N- and C-terminal lysine residues that may account for the ability of PTN to bind tightly to heparin (2, 4) and extracellular matrix (2). PTN has nearly 50% amino acid sequence identity and perfect conservation of 10 cysteine residues with midkine (MK) (2), a recently isolated retinoic acid-induced heparin-binding differentiation factor (14, 15), and together, PTN and MK establish a unique family of

heparin-binding growth/differentiation factors (2) that is believed to have a broad role in both growth and differentiation.

PTN transforms NIH 3T3 cells (16), and its transcripts have been identified in many tumor specimens and tumor cell lines (10, 17). Recently, a mutant PTN that contains only the first N-terminal 40 aa (PTN 1–40) was shown to be a dominant-negative effector of PTN signaling, because its expression effectively blocked transformation of NIH 3T3 cells by wild-type (wt) PTN and formed disulfide linked heterodimers with wt PTN when expressed in human breast cancer (MDA-MB-231) cells that express high levels of endogenous PTN. Furthermore, PTN 1–40 effectively reversed the malignant phenotype of these cells, indicating that it functionally blocks endogenous PTN signaling and that endogenous PTN signaling is required to maintain the malignant phenotype of MDA-MB-231 cells (18).

MATERIALS AND METHODS

Generation of Highly Chimeric Mice Carrying a *Ptn* Dominant-Negative Mutation Allele. The fragments of the mouse *Ptn* gene were isolated by screening a λ Dash (Stratagene) mouse genomic library derived from the 129/SV mouse strain, by using a 350-bp *Sma*I coding region fragment (base pair 1–325) of mouse *Ptn* cDNA as a probe, and a single positive clone was subcloned into pBluescript II SK(+) plasmid vector (Stratagene). Restriction mapping, DNA sequencing of coding portions and exon-intron junctions, and hybridization with exon-specific oligonucleotide probes were used to construct the restriction map shown in Fig. 1A. To introduce the *Ptn* dominant-negative construct into the endogenous mouse *Ptn* gene, a *Ptn* gene targeting vector was prepared as follows. A 4.5-kb *Kpn*I fragment of the mouse *Ptn* gene (including intron 2, exon 3, intron 3, exon 4, and intron 4) was subcloned into the *Kpn*I site of pBluescript II SK(+), yielding a 4.5-kb pBluescript II plasmid. This plasmid was cut with *Sma*I and blunt-ligated with a *Hind*III–*Xho*I fragment containing PGK/neomycin gene from pPGK-Neo-bpA digested by *Hind*III and *Xho*I. After restriction mapping, the resultant plasmid with the PGK promoter 5' to the exon 2 of mouse *Ptn* gene was designated as p*Ptn*4.5/Neo. The p*Ptn*4.5/Neo was cut with *Hind*III–*Bam*HI, filled in, and blunt-ligated to itself to generate p*Ptn*4.5/Neo-blunt. The plasmid p*Ptn*4.5/Neo-blunt was digested by *Kpn*I and blunt-ligated with an *Xho*I–*Hind*III fragment containing pHSV-TK derived from the plasmid pMCI-TK cut by *Xho*I and *Hind*III. This new plasmid was named pm*Ptn*-1. The 10.5-kb *Eco*RI fragment from the mouse *Ptn* λ genomic clone was assembled into pBluescript II SK(+) with the 5' end ligated to the T3 promoter. This resultant plasmid is called p*Ptn*-10.5. The 10.5-kb *Xho*I–*Not*I fragment

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Abbreviations: PTN, pleiotrophin; MK, midkine; ES, embryonic stem.
*Present address: Amgen, Thousand Oaks, CA 91360.

†To whom reprint requests should be addressed. e-mail: TDEUEL@BIDMC.HARVARD.Edu.

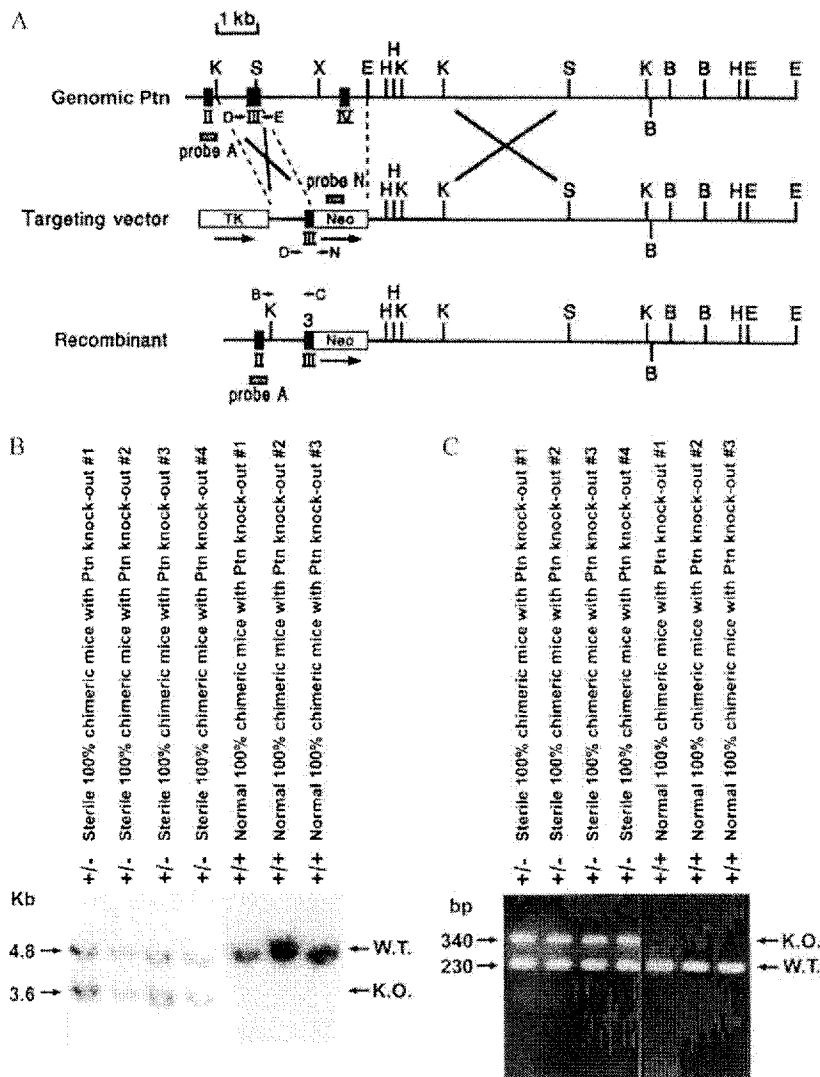


FIG. 1. Targeted dominant-negative mutation of mouse *Ptn* gene. (A) Restriction map of a part of the mouse *Ptn* gene, the targeting vector, and the structure at the mutated locus following the homologous recombination. The coding exons are depicted by black boxes and are numbered. Genomic fragments from the *Ptn* gene and neomycin gene used as probes for Southern blotting are depicted as hatched boxes (probe A and probe N); arrows denote PCR primers (B, C, D, E, and N) used for genotyping the ES cells and the chimeric animals. Neo, the neomycin transferase gene; TK, thymidine kinase gene; arrows depict the transcriptional orientation of these genes. Only relevant restriction sites are shown: K, *Kpn*I; S, *Sma*I; X, *Xho*I; E, *Eco*RI; H, *Hind*III; B, *Bam*HI. (B) Southern blot analysis of genomic DNA extracted from the tails of the sterile, highly chimeric mice with a *Ptn* dominant-negative mutation and normal highly chimeric mice without *Ptn* gene disruption. The DNA was digested with *Eco*RI and hybridized with probe A. The sizes of wt (W.T.) and disrupted (K.O.) alleles are shown. (C) PCR amplification products of the DNA extracted from the mouse tails. The sizes of amplification products of wt (W.T.) and disrupted (K.O.) alleles are shown.

derived from p*Ptn*-10.5 cut by *Xho*I and *Not*I replaced the 3-kb *Xho*I-*Not*I fragment of pm*Ptn*-1, yielding a *Ptn* gene targeting vector.

The *Not*I-linearized targeting vector was electroporated into E14 (kindly provided by D. Y. Loh, Washington University, St. Louis) and RW4 cells [the RW-4 embryonic stem (ES) cell line was established from strain SV129 and kindly provided by R. L. Wesselschmidt and T. J. Ley, Washington University, St. Louis]. G418 and gancyclovir double-resistant ES clones were selected, expanded, and frozen. The homologous recombination event is predicted to replace a 2.8-kb *Sma*I-*Eco*RI fragment of the *Ptn* gene with a neomycin-resistance gene. DNA was isolated from 280 double-resistant E14 ES cell clones and 120 RW4 ES cell clones, digested with *Eco*RI, blotted onto Hybond-N membranes, and probed with the 250-bp *Not*I-*Kpn*I fragment of the *Ptn* gene. The probe hybridizes to a 4.8-kb *Eco*RI fragment derived from the wt allele, whereas the targeted locus is predicted to yield a 3.6-kb fragment. Of the 28 E14 clones and 14 RW4 clones heterozygous at the *Ptn* locus, 6 and 8, respectively, were expanded, and their genetic status was validated. The presence of additional integration sites was ruled out by probing *Eco*RI digests of their genomic DNA with a *Pst*I-*Pst*I neomycin-resistance gene probe. Genomic DNA from the positive clones was also analyzed by using PCR. E14 wt ES cells, RW4 wt ES cells, both G418 and gancyclovir double-resistant E14 clones and RW4 clones without *Ptn* gene disruption, three *Ptn* heterozygous

E14 clones, and eight *Ptn* heterozygous RW4 clones were injected into blastocyst-stage C57BL/6 mouse embryos (~15 cells per blastocyst) and implanted into pseudopregnant ICR females. The resulting chimeras were bred with C57BL/6 mice. For the genotyping, genomic DNA was isolated from the tails of chimeric animals, digested with *Eco*RI, and analyzed by Southern blotting using probe A or by PCR. The *PTN*-specific primers were: D, 5'-AAA AAA AGG TGA AAA AGT CTG ACT GTG GAG-3'; E, 5'-CTC CAA ACT GCT TCT TCC AG-3'; and a neomycin-resistance gene-specific primer N, 5'-CAC GAG ACT AGT GAG ACG TG-3').

Northern Blot Assay. Total RNAs were isolated from testis tissue by the guanidinium-thiocyanate method as described (19). Twenty micrograms of total RNA was separated on 1.2% formaldehyde-agarose gels, blotted onto nylon membrane (Micron Separations, Westboro, MA), prehybridized, and hybridized with an [α -³²P]dCTP-labeled *Sma*I fragment of mouse *Ptn* cDNA. After overnight hybridization, blots were washed twice with 0.5× SSC and 0.1% SDS for 20 minutes at room temperature, washed once for 20 minutes at 68°C, and autoradiographed with intensifying screens at -70°C.

Fertility of the Chimeric Male Mice. C57BL/6 female mice were mated with chimeric males generated from different *Ptn* heterozygous ES clones, wt ES cells, *Ptn* targeting vector randomly integrated ES clones. In the morning, plugs from mated C57BL/6 female mice were harvested and placed in Eppendorf tubes with 100 μ l of PBS, suspended, and smeared

onto glass plate. Spermatozoal quantity and quality were examined by using a microscope. Meanwhile, each highly chimeric male was bred with four C57BL/6 female mice for 4 months to test fertility.

Anatomy Pathological and Histological Examination of Mice. Tissues were collected from euthanized mice for gross and histological examination, fixed in formalin, processed, and embedded in paraffin. Sections (6 μ m) were cut and stained with hematoxylin and eosin by using standard procedures.

Immunocytochemistry. Sections of testes of sterile and fertile chimeric mice were stained with the Apop Tag Plus-Peroxidase (Oncor, catalog no. S7101) to detect apoptotic cells *in situ* as described (20) according to the manufacturer's instructions. The stained sections were mounted and observed by using a light microscope after counterstaining with 1% methyl green.

***In Situ* hybridization.** *In situ* hybridization was performed on paraffin sections of testes from the sterile and fertile males as described (21). Standard protocols for collecting, embedding, and sectioning the tissues were used. pBluescript II mouse *Ptn* cDNA and pPGK-Neo-bpA were linearized with *Eco*RI and *Nco*I, respectively (22), and antisense transcripts were generated by using T7 polymerase. Specimens were hybridized with [³⁵S]thio-UTP-labeled riboprobes. Both pBluescript II mouse *Ptn* cDNA and pPGK-Neo-bpA were also cut by *Xho*I and *Xba*I, respectively, and sense probes were produced by T3 polymerase and used as controls.

RESULTS

Generation of Highly Chimeric Mice Carrying a *Ptn* Dominant-Negative Mutant. To determine the roles of PTN signaling *in vivo*, a *Ptn* dominant-negative mutant allele that encodes PTN amino acid residues -32 to 40 (referred to as PTN 1-40, after removal of the signal peptide) was generated in mice by introducing a *Ptn* mutant targeting vector into ES cells through homologous recombination. The *Ptn* targeting vector (Fig. 1A) was introduced into both 280 E14 and 120 RW4 ES cell lines, and the transfected cells were selected. Southern blot and PCR analysis of DNA established that 28 E14 clones and 14 RW4 clones were heterozygous at the *Ptn* locus. Three E14 *Ptn* heterozygous ES clones, eight RW4 *Ptn* heterozygous ES clones, one positive-negative selected E14 clone without mutation of the *Ptn* gene (as control), one positive-negative selected RW4 clone without targeting of the *Ptn* gene (as control), and E14 wt cells and RW4 wt cells (as controls) were expanded and injected into mouse blastocysts. All highly chimeric founder mice were analyzed. Genotypes were identified by Southern blot and PCR amplification of tail DNA (Fig. 1B and C).

Fertility and gross pathological examination of the chimeric male mice. Male mice with >90% chimerism were bred but failed to produce offspring. For this reason, mice were tested for fertility over a 6-month period, including 30 males with the dominant-negative *Ptn* mutation produced from 11 ES clones (including both E14 and RW4) heterozygous at the *Ptn* locus, 4 males generated from double-selected E14 and RW4 cells without disruption of the *Ptn* gene, and 4 males derived from E14 and RW4 wild-type ES cells. Of the 30 male mice with the *Ptn* dominant-negative mutation, 22 failed to sire offspring. However, each produced many vaginal plugs in multiple matings with B6 female mice. The 8 remaining highly chimeric

male mice (derived from 3 *Ptn* heterozygous RW4 clones and 1 *Ptn* heterozygous E14 clone) were fertile, but none of these were established as germ-line transmission as indicated by inheritance of agouti color and genotyping (data not shown). When the control highly chimeric male mice (generated from wt E14 or RW4 cells and from the double-selected E14 or RW4 clones that lacked the homologously recombined *Ptn* gene) were bred with B6 females, all sired agouti offspring, indicating germ-line transmission.

Although the sterile male chimeric mice with the *Ptn* dominant-negative mutation produced vaginal plugs, sperm were never detected. The testes of these sterile chimeric mice were normally descended in the scrotum and the genital ducts (i.e., epididymis, vas deferens) and accessory glands (i.e., seminal vesicle, prostate and bulbourethral glands) were macroscopically and histologically normal. The remainder of the testis was markedly atrophic, and the testes of the sterile male chimeric mice weighed only \approx 10% that of the normal control animals and those of the fertile chimeric males (i.e., the mice with the PTN dominant-negative mutation that failed to establish germ-line transmission) (Table 1).

Detection of *Ptn* and Its Mutant Gene Expression. Expression of the dominant-negative mutant gene in chimeric mice was confirmed by Northern blot analysis of total RNA extracted from testes of sterile males (see below) in which both the 1.5-kb endogenous *Ptn* mRNA species and a larger *Ptn* transcript of 2.8 kb generated from the disrupted *Ptn* gene were found (Fig. 2). The larger transcript uses the polyadenylation site of the neomycin-resistance gene that was fused with the *Ptn* gene at the *Sma*I site in the middle of exon 3. The fidelity of the *Ptn* transcript encoded by the mutant *Ptn* gene was validated by using reverse transcription-PCR and DNA sequence analysis (data not shown). It encodes 118 aa to include amino acid residues -32 to 40 of PTN and the additional 46 amino acids encoded by the PGK promoter region terminated by a stop codon in the pPGK-neomycin cassette. Western blots with a chicken polyclonal anti-PTN antibody detected the mutant *Ptn* gene product only in high chimeric mice carrying the *Ptn* dominant-negative mutation and not in the control, highly chimeric mice from normal control ES cells that were without disruption of the *Ptn* gene (data not shown).

We also examined the pattern of expression of the neomycin gene in the testes of the sterile and the fertile chimeric mice with the *Ptn* dominant-negative mutation by using *in situ* hybridization. The transcripts of the neomycin gene were found only in the sterile chimeric mice, indicating that the spermatogonia of the sterile chimeric males with the PTN dominant-negative mutation generated from ES cells contained the homologously recombined *Ptn* gene. The results also indicated that the spermatogonia of the fertile chimeric mice were not derived from ES cells containing the PTN dominant-negative mutation (Fig. 3A-C).

To test whether spermatocytes express the endogenous *Ptn* gene during spermatogenesis, we used a mouse *Ptn* antisense riboprobe to examine *Ptn* gene expression in the seminiferous epithelium from sterile and fertile chimeric mice with the *Ptn* dominant-negative mutation by using *in situ* hybridization. The results indicate that production of *Ptn* mRNA is more intense in the seminiferous epithelium of the sterile mice than the normal mice. It is possible that the more intense hybridization signal seen in the seminiferous epithelium of the sterile mice (Fig. 2) may reflect greater mRNA stability in these animals.

Table 1. Characteristics of chimeric PTN mutant mice

Reproductive status	Body weight, g	Testis, mg	Epididymis, mg	Seminal vesicle, mg	Epididymis sperm count, $\times 10^6$
Fertile	33 \pm 5	138 \pm 18	38 \pm 5	98 \pm 16	19 \pm 3
Sterile	33.5 \pm 6.8	22 \pm 9	20 \pm 3	95 \pm 11	0

Values given are mean \pm SD of six fertile or six sterile chimeric mice.

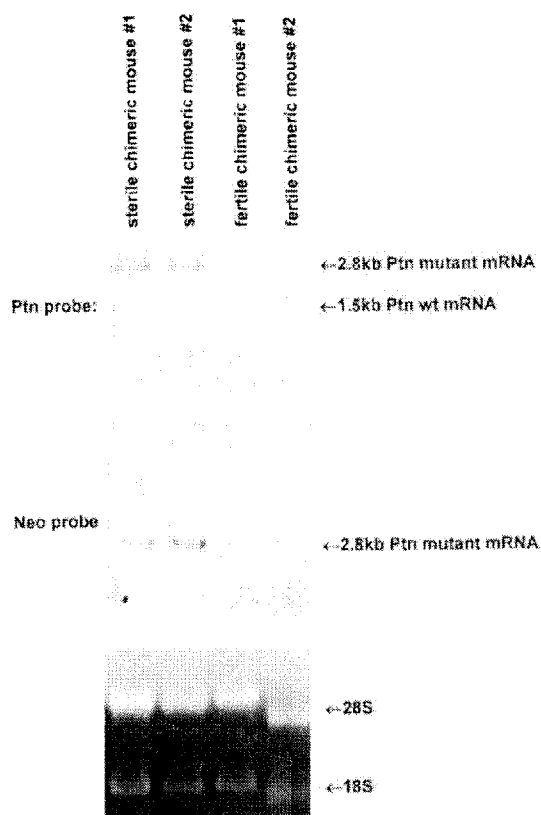


FIG. 2. Northern blot analysis of total RNA prepared from the testes of the sterile and the fertile chimeric mice with a *Ptn* dominant-negative mutant allele. The blot was probed with a *Sma*I fragment of mouse cDNA. Bands corresponding to *Ptn* transcripts are indicated.

The cDNA containing the *Ptn* dominant-negative mutant allele was fused to the neomycin gene at the 3' end and does not contain three ATTTA repeats implicated in mRNA instability that are found in wt PTN 3' untranslated mRNA. *Ptn* gene expression was present in spermatocytes during all stages of spermatogenesis, although the intensity of staining was lower in the normal fertile chimeric mice than in the sterile animals, suggesting that PTN signaling may be important in spermatogenesis (Fig. 3 D–F).

Histological Examination and Analysis of Apoptosis. Histological examination revealed that the epididymis of the adult wt mice and the fertile chimeric mice were packed with sperm (Fig. 4A); in striking contrast, differentiated spermatozoa were not seen in the epididymides of adult sterile mice (Fig. 4B). A thorough histological analysis of the testis was then done; spermatogenesis and spermiogenesis in 10 of the highly chimeric 2- to 4-month-old fertile male mice (including mice derived from wt E14 cells or RW4 cells, from double-selected E14 or RW4 clones without disruption of the *Ptn* gene, and from the *Ptn* mutant heterozygous E14 and RW4 cell clones) occur normally in the seminiferous tubules (Fig. 4C).

However, degeneration of the seminiferous epithelium was observed in all 10 highly chimeric 2- to 4-month-old sterile male mice (generated from E14 or RW4 clones heterozygous for the *Ptn* dominant-negative mutation). In these mice, the number and diameter of the seminiferous tubules were markedly reduced, and primary spermatocytes were present in the seminiferous tubules but the number of these germ cells was dramatically reduced and the primary spermatocytes themselves were degenerate with dark, pyknotic nuclei and occasional karyorrhexis (Fig. 4D). The cytoplasm of the primary spermatocytes was variable and stained eosinophilic to slightly basophilic. Some of the primary spermatocytes formed large

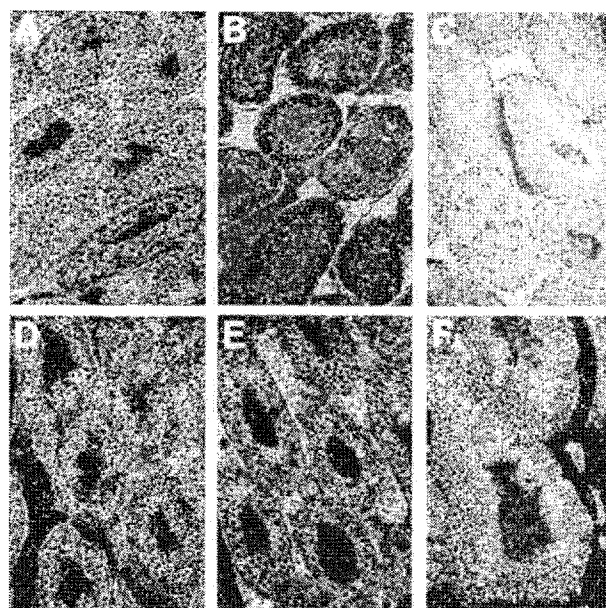


FIG. 3. Expression of neomycin and *Ptn* mRNA in the testes of the sterile and fertile chimeric males with a targeted *Ptn* dominant-negative mutant allele. (A) Neomycin gene transcript was detected in all kinds of germ cells during spermatogenesis in the sterile chimeric mice using neomycin gene antisense as a riboprobe. (B) The signal was absent in sections of testes from the sterile animals when neomycin sense was used as a riboprobe. (C) The neomycin gene signal was absent in sections of testes from the fertile chimeric by using a neomycin gene antisense riboprobe for detection. (D) Intensive expression of *Ptn* mRNA in seminiferous epithelium was found in sections of testes from the sterile chimeric males by using mouse *Ptn* antisense as a riboprobe. (E) The signal was absent in the sterile animals by using a *Ptn* sense riboprobe. (F) *Ptn* signal was detected in the testes of the fertile chimeric mice with a mouse *Ptn* antisense riboprobe.

multinucleated cells. Staining with Apop Tag Plus-Peroxidase identified striking clusters of apoptotic germ cells in the sterile males as compared with the extent of cell death known to occur in normal spermatogenesis (Fig. 4E and F). In some of the tubules, spermatogonia and spermatocytes showed vacuolation and ballooning of the cytoplasm and focal loss of all germ cells. Neither identifiable secondary spermatocytes nor mature sperm were observed in any of the seminiferous tubules examined. However, the interstitial (Leydig) cells appeared normal.

We also compared 10 sterile chimeric males carrying the *Ptn* dominant-negative mutation with their normal counterparts between 6 and 8 months of age. These mice had the same abnormalities in the seminiferous tubules as did 2-month-old males. In addition, the number of seminiferous tubules was markedly reduced; the seminiferous tubules that were present were lined with a single layer of degenerating spermatogonia with pale nuclei, large nucleoli, and indistinct nuclear membranes. The cytoplasm was basophilic, slightly granular, and contained numerous small clear vacuoles or large single vesicles (Fig. 4G). Spermatocytes could not be definitively identified and mature sperm were not observed. In contrast to the atrophied seminiferous tubules, the interstitial cells had proliferated dramatically (Fig. 4H). None of these abnormalities were found in any of the highly chimeric mice with the *Ptn* dominant-negative mutation that were examined at 2 weeks of age (data not shown). We examined the remaining organs and tissues of the sterile animals; no abnormalities were found in brain, pituitary, lung, heart, gastrointestinal tract, bone marrow, skeletal muscle, accessory sex glands, thymus, spleen, lymph nodes, adrenal glands, urinary bladder, or kidney (data not shown).

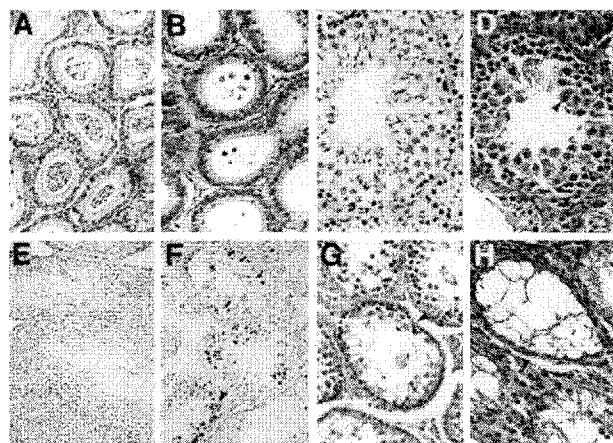


FIG. 4. Histology and immunohistochemical staining of the epididymis of adult sterile and fertile chimeric mice with a *Ptn* dominant negative mutation. (A and B) Hematoxylin and eosin-stained sections of epididymis of the fertile and sterile animals. The epididymis of the fertile male mice are packed with spermatozoa (A), whereas those of the sterile male mice lacked detectable spermatozoa (B). (C and D) Hematoxylin and eosin-stained section of the fertile and sterile chimeric mouse testes. Normal spermatogenesis was seen in the seminiferous tubules of the 2- to 4-month-old fertile chimeric mice (C), and degeneration of seminiferous epithelium was observed in 2- to 4-month-old sterile chimeric animals generated from *Ptn* dominant-negative mutation heterozygous E14 or RW4 clones (D). The multinucleated giant cells, as noted by arrowheads, and an expanded layer of cells, indicated by a double-headed arrow, have a nuclear morphology characteristic of premeiotic cells. (E and F) Testis sections of sterile and fertile chimeric mice were stained with the Apop Tag Plus Peroxidase Kit to detect apoptotic cells *in situ*. The assay shows clusters of apoptotic germ cells and a marked increase in apoptosis in the testes of the sterile mice (F) as compared with the extent of cell death known to occur in normal spermatogenesis (E). The labeled cells are denoted by an arrow. (G and H) In older mutant males, progressive degeneration of the germinal epithelium occurred, ending with the formation of striking vacuolization of tubules that were observed in testis from the sterile males at 6 months (G) and 8 months (H). Sertoli cells and Leydig cells appear normal and are denoted by arrows.

DISCUSSION

PTN is a weak mitogen for endothelial (3, 14, 16), epithelial (3, 13), and fibroblastic cells (1, 2). PTN also induces differentiation of oligodendrocyte progenitors and neurite outgrowth (2, 3, 23; H.-J.Y. and T.F.D., unpublished data). The results of this study suggest that PTN is important for spermatogenesis in the mouse. In this work, we demonstrate that interruption of PTN signaling by the introduction of the dominant-negative mutant into germ line of mouse leads to apoptosis of spermatogonia in all stages of development. Growth factor withdrawal from dependent cell lines is known to lead to apoptosis in transformed mammalian cell lines (23, 24). However, platelet-derived growth factor-stimulated fibroblasts become apoptotic when serum is withdrawn but not in the presence of insulin, suggesting that growth factors, in this case PTN, and other cytokines are needed to suppress apoptosis in specific settings.

PTN is widely expressed in different cell types and at different times in neuroectoderm and mesoderm during embryogenesis. However, in adult tissues, PTN is expressed only at low levels in some cells in the brain, bones, gut, uterus, and ovary, but, in testis, PTN expression levels are significantly higher (3, 5, 23). It is possible that the process of continuous self-renewal and differentiation in developing spermatogonia make them uniquely susceptible to loss of PTN signaling that results from this mutation and that other growth factors functionally compensate in other tissues.

The PTN dominant-negative mutant gene encodes a polypeptide of 40 residues (after cleavage of the signal peptide) that forms

inactive heterodimers (18) with the wt PTN associated with loss of PTN signaling. However, it is possible the PTN 1–40 forms heterodimers with the wt product of the MK gene (14, 25, 26), a protein that shares nearly 50% amino acid identity and perfect conservation of cysteines with PTN (2), and has similar functional activities as PTN as well as being highly expressed during development. PTN 1–40 heterodimerizes with MK itself, because there is complete conservation of 3 cysteine residues in the first 40 aa of both proteins. However, it is equally possible that PTN 1–40 interferes with the normal interactions of wt PTN and a second protein of vital importance in spermatogenesis. This report demonstrates the unique importance of a single growth factor in the regulation of spermatogenesis in mice. Because abnormal spermatogenesis is a feature of many cases of infertility in human males (27, 28), the mice generated in this study may be useful models to study infertility in human males.

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